Neural and marrow–derived stromal cell sphere transplantation in a rat model of traumatic brain injury

DUNYUE LU, PH.D., YI LI, M.D., ASIM MAHMOOD, M.D., LEI WANG, M.D., TAHIR RAFIQ, M.D., AND MICHAEL CHOPP, PH.D.

Departments of Neurosurgery and Neurology, Henry Ford Health Sciences Center, Detroit; and Department of Physics, Oakland University, Rochester, Michigan

Object. This study was designed to investigate the effect of treatment with a novel composite material consisting of embryonic neurospheres and bone marrow–derived stromal cell spheres (NMSCSs) in a rat model of traumatic brain injury (TBI).

Methods. The NMSCS composite was injected into the TBI contusion site 24 hours after injury, and all rats were killed on Day 14 after the transplantation. The Rotarod test and the neurological severity score were used to evaluate neurological function. The transplanted NMSCS was analyzed in recipient rat brains by using histological staining and laser scanning confocal microscopy. The lesion volumes in the brains were also calculated using computer image analysis.

Conclusions. Rats that received NMSCS transplants had reduced lesion volume and showed improved motor and neurological function when compared with control groups 14 days after the treatment. These results suggest that transplantation of this novel biological material (NMSCS) may be useful in the treatment of TBI.

KEY WORDS • traumatic brain injury • transplantation • rat

TRAUMATIC brain injury causes loss of brain tissue and impairment of neurological function. Fetal brain tissue, embryonic stem cells, neural stem cells, marrow–derived stromal cells, and other genetically engineered cells have been transplanted into brain in an effort to reduce the neurological functional deficits associated with CNS diseases and injury. Among many variables, the success of tissue transplantation interventions depends heavily on the survival of the graft material. Survival of grafts in the brain may be promoted by reduction of neuronal cell death triggers (hypoxia, hypoglycemia, and growth factor deprivation) and the addition of new transplantation procedures and new tissue sources. For example, a major limiting factor for the use of neural transplantation as a treatment of Parkinson disease is that only 3 to 20% of grafted dopamine neurons survive the procedure. The addition of basic fibroblast growth factor and members of the transforming growth factor–β superfamily, such as glial cell line–derived neurotropic factor, significantly improve the outcome of nigral transplant. Inhibitors of apoptosis such as caspase inhibitors and agents counteracting oxidative stress also significantly increase the survival of grafted dopamine neurons.

Embryonic brain tissue has been used to treat experimental brain injury, but only a small percentage (< 20%) of the embryonic transplants survived when transplanted immediately after brain injury. Although delayed (10–14 days after brain injury) transplantation increases survival (80%), a second craniotomy for transplantation 10 to 14 days after brain injury is needed. A study of combined graft materials comprising muscle cells and mesencephalic tissue transplanted into hemiparkinsonian rats showed that in vivo, neonatal muscle cells secrete factors that promote survival and/or outgrowth of fetal midbrain dopamine cells and improve the levels of tyrosine hydroxylase in grafted striatum. Recently, MSCs have been studied in vitro and used as a cell source to treat CNS disease and other organ disorders in vivo. Our previous studies showed that MSCs administered intravenously improve neurological outcome in rats after TBI, and these cells express neuronal and astrocytic markers in the brain.

When cultured, fetal brain tissue can form neurospheres, which have been transplanted into brain to treat stroke in animals (unpublished data). Because fetal brain provides a supportive microenvironment for proliferation and differentiation of neural cells and MSCs express an array of growth factors, we hypothesized that MSCs cocultured with neurospheres from fetal brain tissue may be useful for transplantation. This study was designed to investigate the morphological changes of MSCs when
coclultured with fetal neurospheres in vitro and the effect of neural narrow–derived stromal cell sphere transplantation on in vivo contusion injury in rat.

Materials and Methods

All experimental procedures have been approved by the Care of Experimental Animals Committee of Henry Ford Hospital.

Neurosphere Preparation

Donor cortical tissue was obtained from embryonic Day 13 fetuses in Wistar rats that had been anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital. All fetuses were removed and placed in ice-cold Hank’s balanced salt solution. The brains were removed and the sensorimotor cortical region was dissected out. After all the cortical tissue was harvested, tissue pieces were incubated with 0.1% trypsin in Hank’s balanced salt solution. Cell viability was measured using the trypan blue exclusion method and found to be between 75 and 95%. After culturing in IMDM in a chamber for 2 days, the cells were reharvested, treated again in IMDM, and continued to be cultured for 3 days by which point the spheres had formed. One day prior to mixing with MSCs, 25 μg/ml of DiI was added to the media to allow red labeling of the sphere cells under the fluorescent microscope. Some DiI-labeled spheres were continually cultured for 7 days and used as control.

Preparation of MSCs

The MSCs were harvested from male Wistar rats. After the donor rats were killed using an overdose of ketamine and xylazine, the femurs and tibias were dissected away from attached muscle and connective tissue. The ends of the bones were removed and marrow was extracted by inserting the needle of a 1-ml syringe into the shaft of the bone and flushing it with 1 ml of phosphate-buffered saline. The treated cells were cultured in the flasks containing IMDM. After the cells were incubated for 3 days, the nonadherent cell population was removed by replacing the medium. The adherent layer was washed once with the fresh medium and then was continuously cultured for 10 days. One day before mixing with neural spheres, 30 μg/ml DiO was added to the media to label the MSCs, which were green when observed under a fluorescent microscope.

Neural Cell and MSC Sphere Preparation

After the MSCs were harvested and washed with the media, 30 μl of the cell suspension was taken and mixed with 30 μl of 0.4% trypan blue stain. Viable cells were counted under the phase-contrast microscope by using a hemacytometer and a counter. The MSCs and neural cells were mixed at a ratio of 1:50 and cocultured in IMDM for 7 days before transplantation.

Animal Model

Male Wistar rats were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg of body weight). Rectal temperature was controlled at 37°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in a stereotactic frame, and two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between the lambda and bregma. The second craniotomy allowed for movement of cortical tissue laterally. The dura mater was kept intact over the cortex. Injury was induced by impact on the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm-diameter polyethylene sphere. Injury was induced by impact on the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm-diameter polyethylene sphere.

Transplantation Technique

Twenty-four hours after TBI was induced, the rats were reanesthetized with chloral hydrate. Suspended NMSCs in 20 μl of saline (containing 10 spheres with diameters of 20–100 μm) were stereotactically injected into the center of the contusion injury (3-mm depth from the contusion surface) of rat brain. The rats in the control groups received injections of saline, MSCs, and neurospheres, respectively, 24 hours after TBI. All rats were killed 14 days after transplantation.

Histological Analysis

All rats were perfused intracardially with 0.9% saline, and their brains were removed, embedded in optimal cutting temperature compound, and stored at ~80°C. The brains were cut on a freezing microtome at 20-μm intervals. Every 20th section was stained with hematoxylin and eosin for measurement of the lesion by a computer imaging analysis system.

Laser Confocal Image Acquisition

To observe the relationship of the grafts and the cerebral tissue, the sections were analyzed with an argon/krypton laser scanning confocal imaging system (MRC 1024; Bio-Rad, Cambridge, MA) mounted on a microscope (Carl Zeiss, Inc., Thornwood, NY). The sections from the injured area were screened under a ×10 objective lens at 488 nm for MSC observation (green) and 647 nm for fetal neural cells (red). To investigate the interaction of fetal neural cells with MSCs in vitro, the morphological composition of mixed spheres was analyzed under the laser confocal microscope.

Neurological Functional Evaluation

Neurological motor score measurement was performed using an accelerating Rotarod motor test. Rats were tested before induction of TBI and after TBI on Days 1, 4, 7, and 14 until they were killed. The motor test data are shown as a percentage of a mean of five trials of the Rotarod test compared with the internal pre-TBI baseline values. Before TBI, all rats were evaluated using the NSS. One point was given for failure to perform a task, with a maximum of 14 points. Rats were reevaluated on Days 1, 4, 7, and 14 after the TBI. All measurements were performed by observers blinded to individual treatment.

Statistical Analysis

The NSS and Rotarod tested scores were measured before injury and at 1, 4, 7, and 14 days after TBI. Analysis of covariance for repeated-measures analysis of variance was conducted to test the treatment by time interactions and the effect of treatment throughout time. The paired t-test was used to test the differences among means of lesion size Group I (TBI/saline), Group II (TBI/MSC), Group III (TBI/neurosphere), and Group IV (TBI/NMSCS).

Results

In Vitro Analysis of NMSCs

Using phase-contrast microscopy, a variety of sizes of neurospheres were observed in the control flask. The neurospheres suspended in the medium without obvious processes present in the outer layer moved when the flask was shaken (Fig. 1a). The MSCs, when cocultured with fetal neurospheres, integrated into the neurosphere and formed a mixed sphere. The mixed spheres exhibit numerous long processes surrounding the neurosphere, which adhere to the flask (Fig. 1b). Mixed spheres did not move when the flask was shaken. Using laser-scanning confocal microscopy, cavitation was found inside neurospheres without MSCs at Day 7 (Fig. 2a). In contrast, no necrotic cavitation or necrosis was detected in NMSCs. At Day 4 in coculture, MSCs exhibited long dendrites and processess, and the MSCs appeared to integrate into the neurosphere (Fig. 2b–d). At Day 7, MSCs were distributed in the central suture.
the outer layer of the mixed sphere (Fig. 2e–g). Many MSCs sprouted and exhibited long processes, as shown in Fig. 2h.

Assessment of Graft Material

Fourteen days after induction of TBI, the injured tissue had disappeared and a cavity had formed in the parietal cortex of the left hemisphere (Fig. 3). Using a computer image system, the lesion volumes of all rats were calculated and analyzed. The mean lesion volume (16.8 ± 3.4% of the ipsilateral hemisphere) of the TBI/NMSCS group was significantly decreased when compared with the TBI/saline (23.4 ± 4.8%), TBI/neurosphere (21.1 ± 3.2%), and TBI/MSC (22.6 ± 5.6%) groups (p < 0.05). No significant difference was detected between the TBI/saline group and the TBI/neurosphere group (Fig. 4).

All rats receiving NMSCS had surviving transplants and these grafts appeared to attach to the wall of the lesion cavity (Fig. 5a–c). Some cells labeled by DiI or DiO migrated into the brain parenchyma, and many of these cells derived from the fetal neural cells of the mixed sphere (Fig. 5d). Of the nine rats receiving neurosphere transplantation, only three had surviving grafts. A few MSCs migrated into the parenchyma in rats receiving that transplant material. These data demonstrate that the NMSCSs not only survive well in vitro but also in vivo.

Neurological Functional Evaluation

The Rotarod scores were significantly decreased in all rats 1 day after induction of TBI. The transplantation of NMSCSs into the contusion site of the TBI/NMSCS group promoted improvement in motor function (Rotarod test) at Days 4, 7, and 14; however, only on Day 14 did the level of improvement achieve significance when compared with the other groups (p < 0.05, Fig. 6). The NSSs of the rats receiving NMSCS transplantation likewise improved significantly at Day 14 when compared with all of the other groups (p < 0.05, Fig. 7).

Discussion

The results of this study show that in vitro, MSCs co-cultured with neurospheres integrate into neurospheres and develop long processes. They form a firm sphere without cavitation, in contrast to pure neurospheres in which caviation is evident. This suggests that cells from the neurosphere and marrow stroma synergistically support growth. In vivo, all NMSCS grafts survive and they become attached to the wall of the lesion cavity 14 days after transplantation, in contrast to graft survival in three of nine rats receiving transplanted neurospheres. The transplantation of NMSCSs into the contusion site after TBI significantly...
reduced the lesion volume when compared with the control groups. Among all groups tested, only the TBI/NMSCS group exhibited a significant improvement in NSSs compared with the other groups. These results suggest that NMSCSs are an effective biological material for transplantation to treat TBI.

Many biological materials have been studied for use in transplantation to treat CNS disorders, such as adrenal medullary cell grafts in patients with Parkinson disease and fetal brain tissue grafts to treat brain and spinal cord injury. A variety of cells, including neural stem cells, human postmitotic neurons, and MSCs have been used to treat brain injury. Approximately 80% of the fetal brain tissue grafts die after immediate postinjury transplantation and the remaining neural cells in grafts show loss of processes because of tissue hypoxia. Transplantation of fetal brain tissue 24 hours after brain injury in a rat model of lateral fluid-percussion injury results in a 68.8% graft survival rate in animals only receiving fetal brain tissue and a 62.5% graft survival rate in those also receiving nerve growth factor. Transplantation of fetal brain tissues 10 to 14 days after injury results in an 80% graft survival rate. Graft material survival in brain is an important issue in cell and tissue transplantation. Modification of the microenvironment into which cells and tissue are transplanted may enhance graft survival. Specific neurotransmitter release and growth factor expression in brain may benefit graft survival and enhance neurological function after brain injury. Tissue and cell cocultures and co-grafts are promising materials in neural transplantation research. Mesencephalic cells cocultured with neonatal muscle cells have been shown to increase the number of tyrosine hydroxylase immunoreactive neurons in vitro and in vivo. Combined brain and adult peripheral nerve tissue grafts have been shown to benefit anatomical remodeling and long-term functional recovery in rats with chronic paraplegia. In our study, a 100% graft survival was found in the TBI/NMSCS group, whereas only three transplants survived in the TBI/neurosphere group (nine rats), and only scattered MSCs were detected in the TBI/MSC group. It can thus be inferred that NMSCS and MSC transplant materials may tolerate hypoxia after brain injury.

Rat MSCs cultured in growth factors grow long processes in vitro, and these cells when injected intracerebrally enter injured brain and provide significant improvement in functional outcome compared with MSC transplantation alone (unpublished data). Our results here demonstrate that MSCs integrate into the fetal neurospheres and extend very long processes. The NMSCSs appear to form an integrated biological material, with no evidence of cavitation. Cells of fetal brain tissue express many developmental proteins, such as growth factors. The microenvironment containing these proteins benefits differentiation, proliferation, migration, and connection of neurons during development of the brain. The MSCs also secrete an array of growth factors.
that support the differentiation and proliferation of stromal cells in culture;\textsuperscript{1,4,13} therefore, mixed MSCs and neurospheres interact synergistically. This hypothesis is also supported by the observation that NMSCSs, when placed in the contusion site after 10 days in culture, attach to the wall of the lesion cavity and survive 14 days after transplantation. In contrast, only three surviving grafts were detected in nine rats receiving neurosphere transplantation alone. The lesion volume of the NMSCS-treated group significantly decreased and the neurological function of these animals significantly improved when compared with control groups.

There are several possible mechanisms by which NMSC transplants enhance behavioral recovery in animals. Our study shows that the NMSCSs transplanted 1 day after TBI survive at least 14 days after transplantation, which demonstrates that they may tolerate hypoxia and therefore can be transplanted into a hypoxic lesion early after brain injury. The effects of this graft material on the injured tissue and on the improvement of neurological function may be mediated by protecting injured brain tissue and reducing tissue loss after TBI. Although the NMSCSs lined the wall of the lesion cavity and some migrated into brain, it is unlikely that synaptic connections between the grafted cells and the parenchymal cells were established during this limited time frame. The NMSCSs may also be a new source of cytokines and trophic factors, which may support cell survival and promote brain plasticity; however, the mechanisms that underlie reduction of the lesion volume and improvement in neurological function need further study.

**Conclusions**

We have shown that MSCs cocultured with neurospheres form mixed spheres that contain both neural cells and MSCs with long processes. After transplantation of NMSCSs into the contusion site of brain, the NMSCSs survive, line the cavity of the lesion, and may integrate into the surrounding tissue, which not only reduces lesion volume but also promotes improvement of neurological function in the rat after TBI. These results demonstrate that NMSCS is a new material that may be transplanted into contused cerebral tissue early after brain injury.

**Acknowledgment**

We thank Susan MacPhee for her skillful preparation of this manuscript.

**References**


Manuscript received March 13, 2002.
Accepted in final form June 10, 2002.
This work was funded in part by National Institutes of Health Grant No. NS 42259.
Address reprint requests to: Michael Chopp, Ph.D., Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, Michigan 48202. email: chopp@neuro.hfh.edu.